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Review

Yeast Prions

Evolution of the Prion Concept

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ABSTRACT

Prions (infectious proteins) analogous to the scrapie agent have been identified in *Saccharomyces cerevisiae* and *Podospora anserina* based on their special genetic characteristics. Each is a protein acting as a gene, much like nucleic acids have been shown to act as enzymes. The [URE3], [PSI$^+$], [PIN$^+$] and [Het-s] prions are self-propagating amyloids of Ure2p, Sup35p, Rnq1p and the HET-s protein, respectively. The [β] and [C] prions are enzymes whose precursor activation requires their own active form. [URE3] and [PSI$^+$] are clearly diseases, while [Het-s] and [β] carry out normal cell functions. Surprisingly, the prion domains of Ure2p and Sup35p can be randomized without loss of ability to become a prion. Thus amino acid content and not sequence determine these prions. Shuffleability also suggests amyloids with a parallel in-register β-sheet structure.

THE GENESIS OF THE PRION CONCEPT FROM STUDIES IN MAMMALS

The transmissible spongiform encephalopathies (TSEs) of mammals are inexorably fatal degenerative brain diseases whose etiology has long been debated,[1,2] but are widely believed to be caused by an infectious protein. The unusual radiation-resistance of the scrapie agent[3] generated a flurry of speculation on its nature, including a surprisingly accurate early version of the protein-only hypothesis.[4] It was proposed that an altered form of a cellular protein binds a monomer of the normal form, and in this complex, changes the normal to the abnormal form. This is, in essence, the modern view. The key protein was identified genetically as the Sinc gene of mice controlling scrapie incubation period.[5] However, it was only 18 years later that Sinc was shown to be the gene encoding PrP,[6] the major component of the scrapie agent.[7]

PrP is a nonessential protein[8] located on the cell surface where it is bound by a GPI anchor.[9] Animals lacking the Prnp gene encoding PrP are immune to infection by the TSE agent,[10] showing neither pathology, nor substantial replication of infectivity. PrP from brains of TSE-infected animals is quite protease resistant, compared to the protease-sensistive normal protein. It accumulates significantly in diseased tissues because of reduced turnover. The precise structure of the TSE-form of PrP (called PrP-res or PrPSc) is not known, but it is clearly higher in β-sheet content than the normal protein. Amyloid deposits composed largely of PrP-res are observed in many but not all TSEs. The smallest infectious material is estimated to be a 14 to 28-mer, but most of the infectivity is much larger.[11] The protease-resistance of infectious material also suggests that it is amyloid in form, even if frank plaques are not always seen.

While extensive circumstantial evidence points to the TSEs being prion diseases, with the infectious agent nothing more than an altered PrP, definitive experiments are still not available, and there continues to be some debate on this point. The best evidence to date comes from studies in which amyloid formed in vitro from recombinant mouse PrP89-230 was injected into mice transgenic for PrP89-231. The mice developed a scrapie-like disease, albeit after an inordinately long incubation period, and their brains were infectious for normal mice.[12] The fact that this oft-attempted experiment has so far only worked with amyloid of PrP again indicates that amyloid is indeed the infectious material. Recently, Supattapone's group has demonstrated spontaneous in vitro generation of infectivity using Soto's PCR-like adaptation of Caughey's in vitro PrP-res propagation method. This may be the final proof.

The TSEs are infectious, hereditary and spontaneous. Brain extracts of one animal will readily infect another animal on injection or ingestion. Human hereditary Creutzfeldt-Jakob disease is caused by mutations in the gene for PrP, presumably making it more likely to spontaneously assume the prion form. Spontaneous cases are presumed due to spontaneous formation of infectious amyloid by the normal PrP protein.

Injection of infected brain extract into brains of uninfected animals produces disease with a long, but very characteristic incubation period. The incubation period is much longer for infections across species lines (the 'species barrier'). Distinct TSE strains (or variants) have been defined, with different incubation periods, distinguishable signs and symptoms and biochemical characteristics of the altered PrP. These strain (or variant) characteristics are not due to different PrP sequences, but are thought to reflect different amyloid structures. The TSE strain (variant) also affects the species barrier: while one TSE strain may be unable to cross between a particular pair of species, another may readily do so (reviewed in ref. 13).

DISCOVERY OF INFECTIOUS PROTEINS (PRIONS) IN *S. CEREVISIAE*

When yeast is supplied with a good nitrogen source, such as ammonia, it turns off transcription of the genes encoding the enzymes and transporters (e.g., DAL5, encoding the allantoate transporter) needed to use poor nitrogen sources, like proline or allantoate (reviewed in refs. 14 and 15). This control mechanism is called nitrogen catabolite repression or nitrogen control and is mediated by Ure2p. [URE3] is a nonchromosomal gene whose dominant effect is to derepress these enzymes and transporters.[16] [PSI$^+$] is a nonchromosomal gene discovered as a translational suppressor of nonsense mutations,[17] and Sup35p is a subunit of the translational termination factor.[18,19] The molecular basis of [URE3] and [PSI$^+$] was long a puzzle.

We proposed three genetic criteria to distinguish nucleic acid replicons such as viruses and plasmids from prions[20] (Fig. 1).

Reversible curability. While a virus, plasmid or prion may be curable (efficiently eliminated) by some treatment, a virus or plasmid is not likely to be regenerated de novo in less than geologic time. However, the protein capable of becoming a prion is still present in the cured strain and could spontaneously convert to the self-propagating altered prion form.

Overexpression of the protein increases frequency of prion generation. Overproducing a chromosomally encoded protein will not increase the frequency with which a plasmid or virus arises de novo, but increasing the cellular content of a protein able to become a prion should increase the frequency of prion generation. The change must be self-propagating, and so should take over most of the population of molecules of that protein, converting them to the prion form.

Phenotype relation and gene dependence. For viruses, plasmids and prions, the propagation of the nonchromosomal element always requires the activity of some chromosomal proteins. Prion propagation requires at least the gene encoding the protein. If the prion form of a protein were simply an inactive form of the normal protein, then the phenotype of the prion-carrying strains should resemble that of mutants in the gene encoding the protein. This contrasts with viruses or plasmids conferring a cellular phenotype

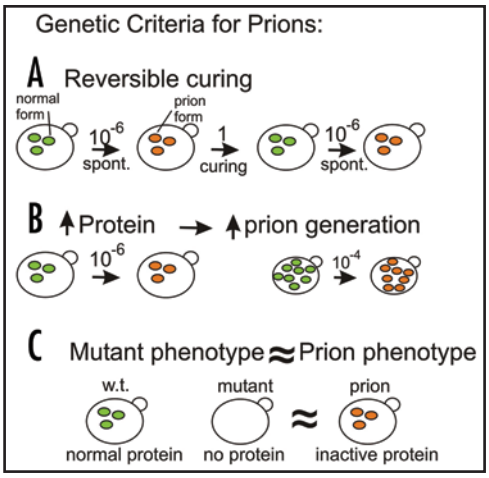

Figure 1. Genetic criteria for prions. Reversible curing means that in a strain cured of a nonchromosomal genetic element, the same element can arise again. Overproducing a protein with the potential to become a prion increases the frequency with which the prion arises. If the prion form of the protein is an inactive form of the protein, then the phenotype of the presence of the prion is the same or similar to that of a mutant in the gene for the protein. Each of these three properties should be characteristic of prions but none of them are known (or expected) for nucleic acid replicons such as plasmids or viruses.

(such as the mitochondrial DNA or killer virus). In these cases, the phenotype of mutation of a chromosomal gene needed for propagation of the nucleic acid replicon is that of absence of the replicon (e.g., killer-negative, glycerol minus).

[URE3] AND [PSI$^+$] ARE PRIONS

[URE3] has all of these properties if viewed as a prion of Ure2p, and [PSI$^+$] qualifies as a prion of Sup35p.[20] [URE3] can be cured by guanidine but arises again at a low frequency.[20] The overproduction of Ure2p elevates the frequency of [URE3] by 20- to 200-fold.[20] Finally, the phenotype of [URE3] strains is very similar to that of ure2 mutants.[21] [PSI$^+$] may be cured by high osmotic strength,[22] but [PSI$^+$] derivatives of the cured strains are easily isolated.[23] Overproduction of Sup35p elevates the frequency of [PSI$^+$] arising de novo,[24] and the [PSI$^+$] phenotype resembles that of sup35 mutants, namely, nonsense suppression.[17]

WHAT DOES IT TAKE TO BE A PRION?

We found that the N-terminal asparagine-rich part of Ure2p was necessary and sufficient to propagate and induce the [URE3] prion,[25,26] and at the same time we reinterpreted the similar results of TerAvanesyan et al. on the Q/N rich N-terminal domain of Sup35p.[27] We call these the prion domains of the respective proteins. It is clear that mutations within the prion domain can affect prion propagation.[28-31] Some of these changes do not prevent the protein from being a prion, but rather introduce a 'species barrier' between molecules.[32,33]

To examine whether there are sequence determinants of prion-formation ability, the prion domains of Ure2p and Sup35p were each shuffled, leaving amino acid content and codon usage unchanged.[34,35] Five shuffled variants of each prion domain were generated and reintroduced into the chromosome in place of the

Figure 2. Scrambled prion domains can still be prions.[34,35] In place of the normal Ure2 or Sup35 prion domains, shuffled prion domains (five of each) with the same amino acid content were constructed and integrated. Each of the shuffled prion domains could be a prion, although one of each was unstable.

normal prion domain (Fig. 2). It was found that each of the shuffled prion domains of Ure2p and of Sup35p were capable of being prions. The frequency of prion formation varied somewhat and in each case, one in five of the shuffled sequences produced only unstable prion variants. But all could be prions.[34,35] In addition, each of the shuffled Ure2p species readily formed amyloid in vitro. In support of this picture, a detailed deletion analysis of the Ure2p prion domain showed that no single region of the prion domain is essential for prion-forming ability.[35]

These results imply that the composition of the prion domain is the critical determinant of prion formation. It is very likely that the high Q/N content of the Ure2p and Sup35p prion domains is important. However, few of the many proteins with such Q/N-rich domains have been found capable of making prions. There are doubtless other compositional features of the prion domains that are important. Their relatively low content of charged residues and hydrophobic amino acids are probably important, but further work will be needed to define the critical features.

Because small deletions in the C-terminal domains of Ure2p[25] and larger deletions of Sup35p[36] C-terminus dramatically increase the frequency of prion formation, it was suggested that the prion domain and C-terminal domains interact, preventing the prion domains from interacting with eachother to form amyloid. However, no evidence for such an interaction could be detected,[37] and the Ure2p prion domain appears to be unstructured in its native (soluble) form. The fact that the prion domain can be shuffled and still support prion formation and propagation argues that if there is such an interaction, it is not important for this process.

SHUFFLEABLE PRION DOMAINS SUGGEST PARALLEL IN-REGISTER STRUCTURE

Although amyloids have long been known to be rich in β-sheet, their more detailed architecture has been unclear. There are at least three mutually exclusive possibilities for the β-sheet architecture of amyloid. An antiparallel β-sheet has adjacent strands bonded to each other running in opposite orientations: N → C next to C → N, for example the amyloid of the Aβ (34–42) fragment.[38] In a parallel in-register β-sheet structure, adjacent bonded strands are in the same orientation: N → C next to N → C, and identical residues are bonded to each other, for example the amyloid of Aβ(1–40).[39-42] Electron spin resonance indicates that amyloids of amylin and of a-synuclein also have parallel in-register β-sheet structure.[43,44] A third possibility is some form of parallel out-of-register β-sheet, for example the β-helix structure of pectate lyases.[45] Here, like the antiparallel structure, nonidentical residues are paired.

Amyloid formation is much like a linear crystal, in that essentially a single species of protein is singled out to join the growing filaments. This specificity demands that there be some specificity in the bonding between chains. For anti-parallel β-sheets or β-helices, this could be large with small, positive with negative, hydrophobic with hydrophobic, hydrogen bonding (donor) with hydrogen bonding (recipient). In these cases, shuffling the sequence would disturb the alignment of complementary residues, and presumably prevent prion formation (Fig. 3).

For parallel in-register β-sheets, hydrogen bonding between Q/N residues[46] or S/T residues, or hydrophobic with hydrophobic

residues could provide specificity. However, charged residues (which are few in the prion domains of Ure2p and Sup35p) should tend to interfere with formation of this structure. Shuffling the residues of a parallel in-register β-sheet does not change the pairing, since identical residues are always paired. Thus, we argue that if a prion domain can be shuffled and still be a prion, it should have a parallel in-register β-sheet structure.[47]

This suggests the sort of model shown in Figure 3B. The core of the amyloid is made up of Ure2p1-65,[48,49] which should be a parallel in-register β-sheet.[47] Indeed Ure2p10-39, a fragment of the prion domain, has been shown to have such an architecture.[50] The folding of the β-sheet is demanded by the diameter of the amyloid filaments of the prion domain.[48] Ure2p66-95 is unstructured in both native and amyloid forms of Ure2p, and we call this the 'tether' (green in Fig. 3B). The C-terminal part of Ure2p apparently does not change its conformation on formation of amyloid.[51,52] A similar parallel in-register β-sheet model can be proposed for Sup35p, since its prion domain is shuffleable and the charged M domain is likely to serve as a tether.

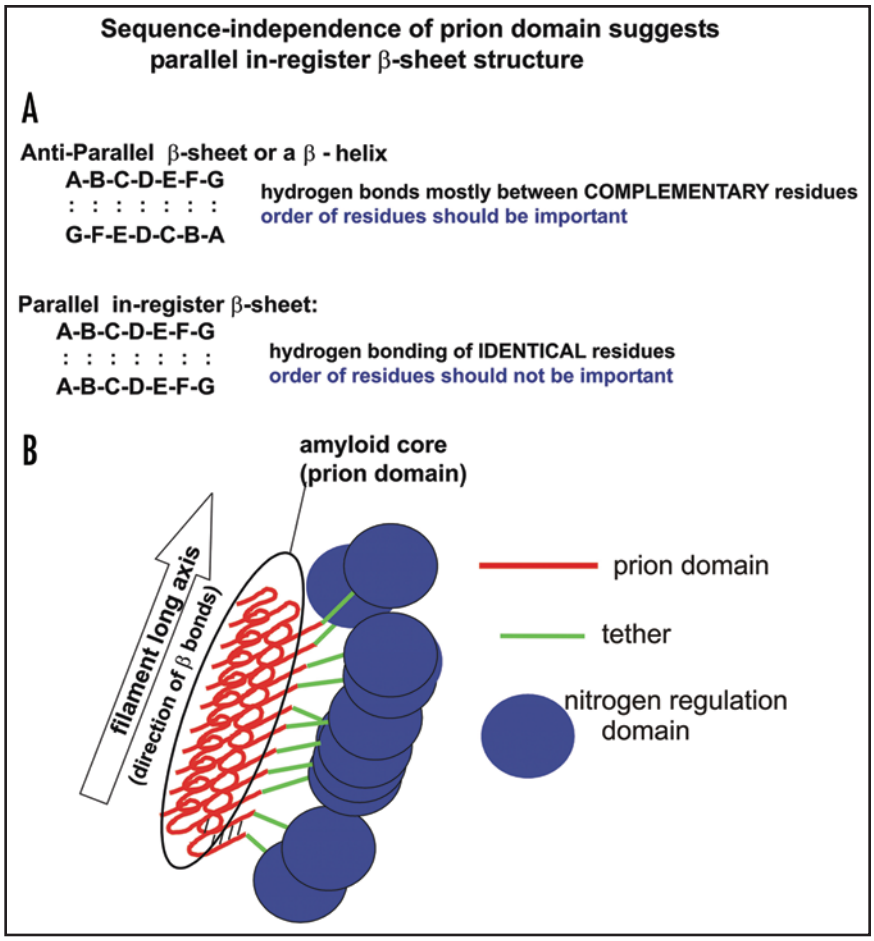

Figure 3. A prion domain insensitive to scrambling should be a parallel in-register amyloid.[47] (A) Nonidentical residues are bonded in an anti-parallel β-sheet or β-helix. The specificity of amyloid propagation (similar to crystal growth) implies that there must be some complementarity of residues. Shuffling such a sequence would destroy any such complementarity and thus prion formation. Shuffling a parallel in-register β-sheet leaves identical residues paired with each other. If a prion domain can be shuffled and not lose prion-forming ability, it suggests a parallel in-register β-sheet structure. (B) Model of Ure2p amyloid structure (see text).

AMYLOID IS THE PRION INFECTIOUS MATERIAL, NOT A DEAD END (SIDE-) PRODUCT

In a ground-breaking study, infection of *Podospora anserina* with the [Het-s] prion by amyloid of recombinant HET-s protein was acheived.[53] Soluble protein was not infectious nor was heat- or acid-denatured aggregated protein. The transmissibility of [PSI+] by amyloid of recombinant Sup35p has also been demonstrated, and evidence was also obtained that the amyloid structure determines the prion variant.[54,55] As mentioned above, amyloid of recombinant PrP has also shown some infectivity for mice.[12]

We have now demonstrated the ability of amyloid formed in vitro from recombinant Ure2p to infect cells with the [URE3] prion[56] (Fig. 4). The low level infectivity of soluble Ure2p (Fig. 4B) is apparently due to filament formation while the experiment is in progress. Cells infected with amyloid of recombinant Ure2p show at least three prion variants, distinguishable by their mitotic stability and by the intensity of their phenotype (degree of DAL5 derepression). Extracts of [URE3] strains are also infectious, and transmit the [URE3] variant that was present in the strain from which the extract was prepared (Fig. 4C). Remarkably, the amyloid made in vitro from recombinant Ure2p is as much as 1/3 as infectious as is an extract (on a per Ure2p molecule basis).[56] The extracts can be used to seed amyloid formation by soluble recombinant Ure2p, but the extent to which this amplification is variant-faithful is limited by the tendency of the 'soluble' Ure2p to spontaneously form amyloid filaments, the latter having a mixture of variant structures.[56]

The Ure2p prion domain by itself, or fused to various other proteins can also form amyloid which is infectious.[56] Cells infected with these fusion proteins (or prion domain alone) show the same spectrum of prion variants as those infected with amyloid formed from the full length protein.

Preliminary size fractionation experiments indicate that infectious material is greater than 20 nm in diameter, indicating a filament length of >40 mer. Amyloid filaments must be sonicated to be infectious, apparently in order to get into yeast. However, while the largest size fraction of filaments has only low infectivity, resonication increases its infectivity many fold.[56] We suggest that this increase in infectivity is a combination of generation of new filament ends (which must be the growing point) and of allowing more facile entry into the cells.

The infectivity of amyloid (and not soluble or other aggregated forms) in all of the prion systems indicates that amyloid is not a dead-end or side product of the prion process. The structure of amyloid formed in vitro has long been recognized to be morphologically heterogeneous. Recently evidence for structural heterogeneity of Aβ amyloid has been obtained.[57] It is clear from the prion studies that prion variants are encoded by differences in amyloid structure. It will be particularly interesting to know what are these structural differences and how they propagate.

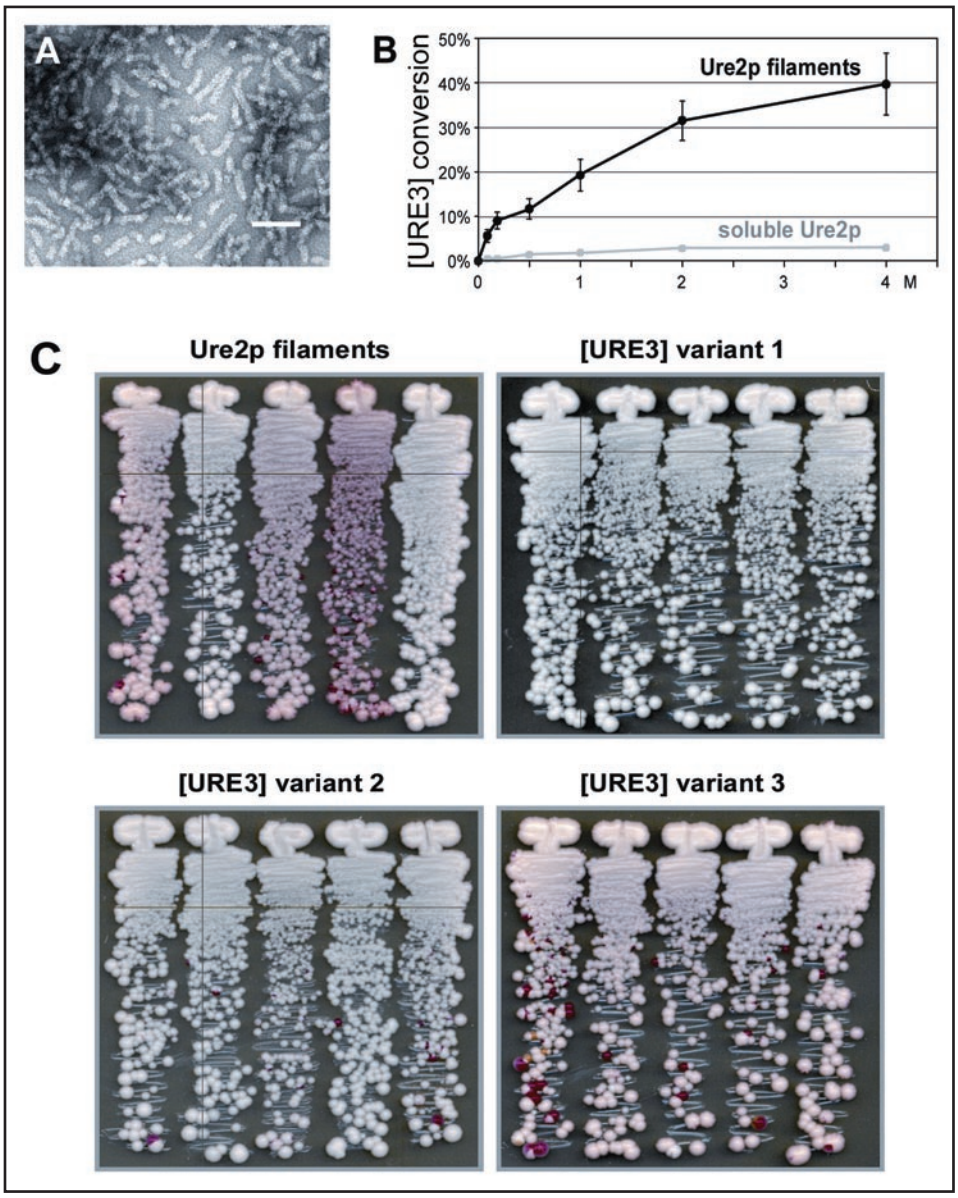

Figure 4. Amyloid of Ure2p is infectious.[56] Amyloid made in vitro from recombinant Ure2p (full length or the prion domain or fusions of the prion domain with other proteins) are infectious for yeast. (A) Filaments are sonicated (bar = 100 nm) and introduced into spheroplasts with a DNA plasmid and polyethylene glycol. (B) A large proportion of the clones transformed for the DNA plasmid were also infected with [URE3]. (C) The infected clones included several prion variants distinguished by stability and intensity of the phenotype, here indicated by activity of a DAL5-promoted ADE2 gene. Red clones have lost [URE3]. Extracts of each variant are infectious and transmit the variant of the strain from which they were made.[56]

[PSI$^+$] AND [URE3] ARE DISEASES OF YEAST

It has been proposed, based on plate tests, that [PSI$^+$] is an advantage to cells carrying it in surviving stress[58] and for evolvability.[59,60] Some strains grow better under certain conditions if they are [PSI$^+$] than if they are [psi$^-$], although there are no conditions that uniformly favor [PSI$^+$], and most conditions favor [psi$^-$].[58-60] The genetic basis for these phenotypes remains to be determined.

All of the conditions were measuring growth, but yeast may be spending most of its time in stationary phase. To what extent are the few conditions favoring [PSI$^+$] represented in the wild? This question is almost impossible to answer directly, and it is further complicated by the fact that whether [PSI$^+$] is favored or unfavored is very strain-dependent.

We examined the distribution of [URE3], [PSI$^+$] and [PIN$^+$] in 70 wild strains.[61] Prions arise de novo and spread by infection, so that even if they are a mild disadvantage to their host, they should be frequently found in the wild. As controls, we examined the distribution of parasitic DNA and RNA replicons of yeast: the 2 micron DNA plasmid, 20S RNA, 23S RNA and the L-BC virus (reviewed in ref. 62). We found that the mildly detrimental nucleic acid replicons were found in varying proportions of the wild yeast (Table 1). For example, 2 micron DNA has been shown to slow growth by 1.5–3.0%,[63] but is found in 38 of 70 wild strains.

We found that none of the wild strains carried either [URE3] or [PSI$^+$] (Table 1). Similarly, [PSI$^+$] was absent from nine clinical isolates,[64] two industrial *S. cerevisiae* and eight other non-*cerevisiae* strains of *Saccharomyces*.[65] This indicates that these prions must be quite substantially detrimental to their host. As previously reported for two clinical isolates, [PIN$^+$] is not rare in the wild (Table 1), but its frequency is similar to the parasitic DNA and RNA replicons, suggesting that it is a rather mild disease.

Our approach measures whether [URE3] or [PSI$^+$] are advantageous or not without addressing specific conditions of growth. It remains possible that there is a natural situation in which [URE3] or [PSI$^+$] are more of a help than a hindrance, just as the mild hemoglobin disease, Sickle Cell Trait, is an advantage in areas where malarial infection is prevalent. However, stress and the need to evolve are daily occurrences for yeast, and if [URE3] or [PSI$^+$] helped in this regard, they would not be hard to find in the wild.

A SELF-ACTIVATING ENZYME ACTING AS A PRION

The word 'prion' means 'infectious protein',[66] and although most prions are found to be self-propagating amyloids, this need not be the case.[4] If an enzyme were made as an inactive precursor, and the active form of the same enzyme were necessary for activation of the precursor, then this could appear as a prion system. The vacuolar protease B of *S. cerevisiae* is made as an inactive precursor, and is normally processed proteolytically to an active form by protease A (reviewed in ref. 67). However, in mutants deleted for protease A (pep4Δ), evidence for some transient self-activation was obtained.[68] We showed that this self-activation of protease B could be propagated indefinitely if cells were grown on nonfermentable carbon sources, under which conditions the gene encoding protease B is derepressed.[69]

Table 1 **Nonchromosomal genetic elements in wild *Saccharomyces***

Nonchromosomal Element	Element Present / Strains Examined
L-A dsRNA virus	15 / 70
L-BC dsRNA virus	8 / 70
20S RNA replicon	14 / 70
23S RNA replicon	1 / 70
2 μ DNA plasmid	38 / 70
[URE3] prion	0 / 70
[PSI +] prion	0 / 70
[PIN +] prion	11 / 70

Seventy wild strains of *Saccharomyces*, including 52 *cerevisiae*, 9 *bayanus*, 9 *paradoxus* isolates, were examined for the presence of the indicated RNA and DNA replicons and prions.[61]

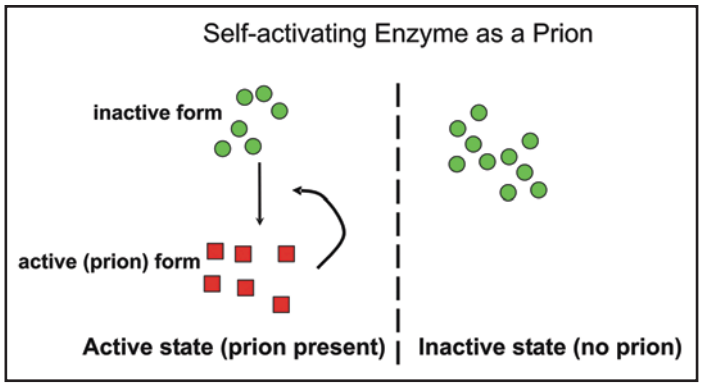

Figure 5. Enzymes needed for their own activation can be prions. "Prion" means "infectious protein", not necessarily amyloid based. If an enzyme is essential for activation of its own precursor, then cells without the active form produce the same as progeny, and those with the active form produce offspring of the same kind. Transmission of just the active form (the protein only) from one cell to another lacking it, transmits the self-propagating activity, and so is an infectious protein. Two such systems have been described, the vacuolar protease B of *S. cerevisiae*,[69] and a protein kinase of *Podospora anserina*.[72]

The inactive state of protease B is very stable, as is the active state. Spontaneous activation of the enzyme occurred only about once in 10^5 cells. Loss of the active state was more frequent, occuring in 1% or more of cells. The active state was transferable by cytoduction, and we called this nonchromosomal genetic element [β].[69]

[β] has all the properties expected of a prion. Growth of cells on glucose media efficiently cures [β], but from cured cells it again arises de novo (reversible curability). Overproduction of the inactive protease B precursor increases the frequency of [β] generation de novo from about 10^{-5} to about 10^{-2} or higher.[69] The propagation of [β] depends on the PRB1 gene, but because the prion in this case is not an inactive form of the protein, the phenotype of [β] cells is the opposite of that of prb1 mutants.

Like the [Het-s] prion of *Podospora anserina*,[70] [β] is a prion with a function for the cells. Without [β], diploid cells fail to undergo meiosis and spore formation, and die more rapidly under starvation conditions.[69] Because [β] is only seen as a prion in the absence of protease A, one could view it as rather artificial. Alternatively, it could be seen as a prion so essential for the cell, that the protease B precursor has evolved to be protease A-cleavable, thus insuring that the prion (active protease B) is never lost. This amounts to duplication of function.

The importance of our findings is that there are many potentially self-modifying enzymes, including protein kinases, protein transacetylases, protein glycosyl transferases, protein methylases, and many others. We suggested that some of these enzymes might become prions under some circumstances. Indeed, we did not have to wait long.

A POSSIBLE PROTEIN KINASE PRION

Crippled Growth is a nonchromosomal genetic element, called [C], of *Podospora anserina*, characterized by slow hyphal growth and dark pigmentation.[71] This trait has recently been shown to require for its propagation a gene encoding a MAP kinase kinase kinase.[72] Most strikingly, overproduction of the same enzyme increases the frequency with which the [C] nonchromosomal genetic element arises.[72] The Crippled Growth phenotype differs from that of mutation of the MAPKKK gene, as expected if it is due to activation of the MAPKKK enzyme, rather than inactivation. Interestingly, the MAPKKK protein has a 60 residue polyQ sequence near its N-terminus, but deletion of this sequence does not impair ability to propagate [C].[72] It is likely that [C] is a self-propagating self-activation of the MAPKKK,[73] but further work will be needed to confirm this conclusion.

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